Effect of Additives and Refrigeration on Reducing Activity, Metmyoglobin and Malonaldehyde of Raw Ground Beef

SUMMARY—The effect of chlortetracycline (CTC), oxytetracycline (OTC), papain, and refrigerator storage temperatures on metmyoglobin (MetMb) reducing activity (MRA) and oxygen utilizing capacity of ground raw beef was investigated. CTC did not affect either MRA or oxygen utilization. OTC showed variable effects on MRA but did not affect oxygen utilization. Papain increased MRA over that of controls and papain treated samples maintained higher MRA longer than controls. Reduction of MetMb took place in the refrigerator at both 0° and 9°C; the rate of MetMb reduction decreased with decreasing temperature. Significant positive correlations were found between MetMb and malonaldehyde in stored refrigerated meats. MRA and MetMb were negatively correlated.

INTRODUCTION

It has recently been shown that DPN related enzyme systems of raw meat are able to utilize oxygen and in the absence of oxygen to reduce MetMb (Watts *et al.*, 1966). The MRA of raw meat varies greatly from sample to sample. It has not yet been established whether there is any relationship between the reducing activity of meat and the rate of MetMb formation during refrigerated storage.

Nor has any correlation been established between MetMb accumulation during storage and unsaturated fat oxidation, although such a correlation might be expected. Keskinel *et al.* (1964) found progressive lipid oxidation during refrigerator storage of raw as well as cooked meats, but they did not attempt to relate lipid oxidation to the oxidation state of the heme pigments. Tims and Watts (1958) and Younathan and Watts (1959) found high values for lipid oxidation in cooked beef where the pigment was in the ferric state, but not in cured meat in which the pigment is in the ferrous state.

Evidence from artificial systems is difficult to interpret because ferrous hemes rapidly oxidize in solutions or emulsions of unsaturated fatty acids. However, Dmitrovskii (1961) reported the initial oxidation rate of codliver oil and vitamin A as being twice as fast for MetMb as for MbO_2. Brown *et al.* (1963), working in pure systems, found induction periods before catalysis began with ferrous pigments; the latter were converted to the ferric pigment before the oxidation rate increased sharply.

MRA is known to be influenced by several additives and environmental factors (Stewart *et al.*, 1965a). It increases with pH in the range pH 5.5–7.0 and with temperatures from 3° to 35°. NaCl inhibits MRA. Chlortetracycline (CTC) has no effect. Oxytetracycline (OTC) has not been tried.

Papain, a proteolytic enzyme, is the principal ingredient used in many tenderizing processes. Meats available on the market which have been treated with papain often appear darker in color than untreated meats, suggesting a preponderance of Mb rather than MbO_2 or MetMb at the meat surface. Proteolytic enzymes might influence meat color in any one of several ways. Denaturation of meat enzymes by the added proteolytic enzymes could decrease enzymatic reduction.

Denaturation of myoglobin would be expected to result in oxidation of the heme moiety to the ferric state; Ross (1939) found that addition of papain to MbO_2 solutions resulted in a brown color after several hours. On the other hand, MRA could conceivably be increased by partial destruction of membranes or formed structures, thus allowing better contact of reactants.

It is even possible that proteolysis could affect the color without a direct effect on the pigment by changing the texture and transparency of the meat. Adjusting the pH of meat to higher values, for example, results in greater light absorbance and a darker color simply because the light is able to penetrate deeper (Stewart *et al.*, 1965a).

This paper describes the effects of CTC, OTC and papain on reducing activity of ground raw beef. The effect of low temperature is further explored to see if reduction takes place over an extended period of time in the refrigerator, even though no reduction could be demonstrated in an hour at this temperature. Possible correlations between the MRA of different samples of meat, the accumulation of MetMb in these samples during refrigerated storage and lipid oxidation in the stored samples are tested.

MATERIALS AND METHODS

Preparation of meat

Locally purchased rib eye of beef was trimmed of fat and connective tissue and ground twice. The beef was thoroughly mixed manually. Fifty g samples were weighed out and stored untreated, or treated with one or more of the following additives: CTC, OTC, NaCl or the proteolytic enzyme, papain. The samples were thoroughly mixed to incorporate additives and packed into glass or aluminum storage cups for spectral analysis. The cups were covered closely with oxygen permeable polyethylene film and stored at 0° to 9°C. Samples for lipid oxidation and MRA assays were formed into balls, covered with film, and stored in polyethylene bags.

Oxygen consumption

Changes of oxygen tension in meat slurries were made with a Beckman polarographic oxygen analyzer as described by Watts *et al.* (1966).

Samples to be assayed for MetMb were removed from the refrigerator, thoroughly mixed in a cold beaker, and returned to the cups. They were covered with a glass cover for reflectance analysis, and the ratio of ferrous to ferric pigments (Stewart *et al.*, 1965b) recorded on a Bausch and Lomb Spectronic 505 Spectrophotometer.

MRA assay

The method was described in detail by Stewart *et al.* (1965a). Samples were flattened in a patty press and warmed for 30 min in a 30°C water bath. Potassium ferricyanide at a level of 0.1% was used for oxidation of all the pigment to MetMb. The sample was packed into cups with a glass cover and assayed for remaining MetMb at the end of a 60 or 90 min period.

Lipid oxidation determination

Two duplicates from a slurry of half of the 50 g sample were distilled by the method of Tarladgis *et al.* (1960). Malonaldehyde in the distillate was determined either by its reaction with 2-thiobarbituric acid or by the ultraviolet method of Kwon and Watts (1963). The results are expressed as mg of malonaldehyde per 1000 g of sample.

Since fairly rapid oxidation of fatty acids can occur in the raw meat slurries, especially after the addition of acid, all slurries were distilled immediately and distillation time was carefully controlled. Even with these precautions, variations in malonaldehyde numbers of less than 0.5 were not considered significant and samples showing these low numbers were averaged and ranked as one sample for statistical tests.

CTC and OTC treatment

The ground beef was treated with 30 ppm of the antibiotics CTC (3 ml per 100 g meat of a standard solution containing 1 mg CTC or OTC per ml of .01*N* HCl) for standard storage studies. In testing their effect on MetMb reducing activity or O_2 consumption, varying amounts of the stock solutions were used.

Papain treatment

In order to show maximal proteolytic activity, papain must be activated (Kimmel and Smith, 1954). All commonly used activating agents are capable of reducing disulfide bonds and chelating heavy metals. Versene, a metal chelator, and compounds such as glutathione or cysteine have been used. It was established that the small amount of activators used did not themselves produce significant differences in MRA.

Activated papain solution was made by mixing one g of finely ground crystalline papain with 50 ml H_2O, 10 ml of 0.01*M* Versene and 1 ml of 0.125*M* cysteine-HCl. The solution was diluted to 100 ml and filtered, several drops of toluene added and the solution stored in the refrigerator at 3°C. The concentration of the stock solution is 0.01 g papain per ml. Papain concentrations of 0.02 to 0.08% of the weight of meat were used.

RESULTS AND DISCUSSION

Effect of CTC and OTC on MRA and O_2 consumption

Table 1 shows results of 5 MRA experiments using both CTC and OTC. Concentrations of CTC used ranged from 20 to 120 ppm. Concentrations of OTC used ranged from 5.0 to 200 ppm. As the table shows, the effects of CTC on MRA were negligible. All values were within the average deviation from the mean of 10% between duplicate controls. One experiment on the effect of CTC on oxygen consumption did not show differences greater than the 7% deviation from the mean of control samples.

Table 1. Effect of CTC and OTC on MRA.

Additive	No. of samples		
	Increase *	Decrease **	Same ***
CTC	0	0	7
OTC	1	5	5

* Increase of $>20\%$ in MRA over that of control.
** Decrease of $>20\%$ in MRA over that of control.
*** Within $\pm 20\%$ MRA of control.

The effects of OTC on MRA varied from sample to sample. Different concentrations used on the same sample of meat showed variable results. Concentrations of OTC in samples that caused variations in MRA did not affect O_2 consumption when slurries were tested from the same lot of meat. These data indicate that OTC is not affecting MetMb reduction through an effect on oxygen utilization.

Little could be found in the literature to suggest the mechanism of the variable effect of OTC on MRA. The additional OH group on the oxytetracycline ring structure may confer on this compound properties of reversible oxidizability in muscle tissues. If so, it might act as an intermediate in the MetMb reduction pathway. Quinones, including menadione, are believed to act as electron carriers between the reduced pyridine nucleotides and ferric hemes. In unpublished work from this laboratory, the addition of various quinones to meat samples both accelerated and inhibited MRA, depending upon concentrations added and the meat sample.

Whatever its mechanism of action, the data indicate that OTC is not a suitable antibiotic for use in experimental studies of enzymatic changes of refrigerated raw beef. Chlortetracycline, which does not have an effect on MRA, is preferable.

MetMb reducing capacity at refrigerator temperatures

In order to determine if reduction of MetMb occurs at refrigerator temperatures, two duplicates of ground beef were allowed to reach the temperatures of storage, 0° and 9°C. The samples were removed from the refrigerator, and the pigments oxidized and mixed in the cold. They were packed into cold glass cups, covered with glass tops, and sealed with masking tape to exclude O_2. After recording initial spectra, the samples were returned to the respective refrigerator temperatures. They were removed

Table 2. MetMb reducing capacity at refrigerator temperatures.

Temperature	No. of samples	Hours required for 50% reduction	
		Range	Average
30°C	5	0.3– 0.9	0.6
9°C	6	2.5– 16.6	7.6
0°C	5	3.7–100	48.0

Table 3. Effect of papain on MetMb reduction with storage.

Storage (days)	MetMb reduction (%) in 90 min.	
	Control	Papain (.04%)
0	88	100
5	52	88
8	29	47

Table 4. Effects of papain on MRA.

No. of experiments	Experiments × storage time	Change in % MetMb reduction of papain treated meat vs. control	
		Range	Average
8	19	0–+82	+39

at intervals and the spectra recorded. Ratios of the duplicates were averaged. Analysis of oxygen utilization was made on slurries of the same lot of meat. The slurries were maintained at the desired temperature by immersion of the flask in a NaCl-ice water bath.

Results of 6 experiments are shown in Table 2. As the data show there were large differences between samples of meat in initial reducing capacity. This has been pointed out by Stewart *et al.* (1965a). Control samples averaged less than one hour to reach 50% reduction of MetMb at 30°C, more than 7 hours at 9°C, and 48 hours at 0°C. In all cases, the samples with highest capacity at 30°C showed highest capacities at the lower temperature. The data show that the same wide variances between muscles occur with oxygen utilization as with MRA. Since rate of MetMb reduction is partially dependent upon oxygen consumption, this would be expected. At 9°C oxygen consumption was slow or not measurable in 10 min. At 0° no measurable O_2 consumption took place in 10 min.

As has been previously mentioned, MRA is not the only factor related to MetMb accumulation. While enzymatic reduction of MetMb is more rapid at 9°C than at 0°C, it would be expected that Mb would oxidize more rapidly at 9° than at 0°C (Urbin and Wilson, 1958). Also, bacterial spoilage occurs more rapidly at 9° than at 0°C in untreated samples, and mold growth is seen at 9°C but not at 0°C in CTC treated samples.

Effect of papain on MRA

Initial experiments showing the effect of different levels of papain on MRA indicate that all levels used (0.02, 0.04, and 0.08%) accelerated MRA. Samples treated with papain also maintained high MRA with storage. A typical set of data is shown in Table 3. Since there was little difference in the amount of acceleration of MRA by 0.04 and 0.08%, and the sample with 0.08% was very mushy, 0.04% was used in subsequent experiments.

Table 4 shows a summary of 8 experiments on the effect of papain on MRA. As the data show, papain accelerates MRA an average of 39% over the activity of the controls. Papain treated samples also maintained their high MRA better with storage than did control samples.

The mechanism of the action of papain is not clear. Data on oxygen consumption in papain treated meats were difficult to interpret, since the oxygen measurements could only be made in meat slurries and it was shown that in such slurries papain brings about rapid destruction of enzymatic activity, probably because of its proteolytic effect on enzymes. It may be postulated that the increased enzymatic activity in papain treated samples is a result of structural changes by proteolysis, resulting in better mixing of the participants in enzymatic reactions.

Correlations between MetMb, MRA and malonaldehyde in stored meat

Statistical evaluation of the results in shown in Table 5. For these statistical comparisons, data from the preceding experiments were used. The values taken for comparison in each experiment were from one storage period only, that which came closest to 4 days. Separate correlation coefficients were obtained on control samples only (samples with no additive except CTC) and on a larger group including samples treated with papain or NaCl. Neither additive skewed the relations significantly.

The highly significant positive correlation between malonaldehyde and MetMb is not necessarily proof that oxidation of the pigment preceded and caused lipid oxidation. The reverse relation is also to be expected; i.e., in the presence of oxidizing lipid, pigments are damaged. In work to be reported at a later date, the addition of lipid

Table 5. Correlation of MetMb, MRA and malonaldehyde in ground beef samples stored 3–5 days in refrigerator.

Values correlated	Composition of sample	r_s[a]	N	Significance level
Malonaldehyde and MetMb	Untreated samples from different animals	0.73	12	.005
Malonaldehyde and MetMb	Control and treated samples from 12 animals	0.63	26	.0005
MetMb and MRA[b]	Untreated samples from different animals	−0.45	11	Not sig.
MetMb and MRA[b]	Treated and untreated samples from 11 animals	−0.44	30	.01

[a] Spearman Rank Correlation Coefficient (Siegel, S., 1956).
[b] MRA compared at 90 min period.

antioxidants to raw meat inhibited not only malonaldehyde but also MetMb formation and may even affect enzymatic activity.

REFERENCES

Brown, W. D., L. S. Harris, and H. S. Olcott. 1963. Catalysis of unsaturated lipid oxidation by iron protoporphyrin derivatives. *Arch. Biochem. Biophys.* **101**, 14.

Dmitrovskii, A. A. 1961. Hematin catalysis of the oxidation of vitamin A and fats. *Biochem. (USSR)* **26**, 202.

Keskinel, A., J. C. Ayres, and H. E. Snyder. 1964. Determination of oxidative changes in raw meats by the 2-thiobarbituric acid method. *Food Technol.* **18**, 101.

Kimmell, J. R., and E. L. Smith. 1954. Crystalline papain. I. Preparation, specificity, and activation. *J. Biol. Chem.* **207**, 515.

Kwon, T. W., and B. M. Watts. 1963. Determination of malonaldehyde by ultraviolet spectrophotometry. *J. Food Sci.* **28**, 627.

Ross, W. F. 1939. The heme-globin linkage of hemoglobin. I. The course of the pancreatic digestion of oxyhemoglobin and of carboxyhemoglobin. *J. Biol. Chem.* **127**, 169.

Siegel, S. 1956. "Non-Parametric Statistics for the Behavioral Sciences," pp. 202–213. McGraw-Hill Book Co., Inc., New York.

Stewart, M. R., B. K. Hutchins, M. W. Zipser, and B. M. Watts. 1965a. Enzymatic reduction of metmyoglobin by ground beef. *J. Food Sci.* **30**, 487.

Stewart, M. R., M. W. Zipser, and B. M. Watts. 1965b. The use of reflectance spectrophotometry for the assay of raw meat pigments. *J. Food Sci.* **30**, 464.

Tarladgis, B. G., B. M. Watts, M. T. Younathan, and L. Dugan, Jr. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. *J. Amer. Oil Chem. Soc.* **37**, 44.

Tims, M. J., and B. M. Watts. 1958. Protection of cooked meats with phosphates. *Food Technol.* **12**, 240.

Urbin, M. C., and G. D. Wilson. 1958. Fresh meat characteristics which influence packaging requirements. *Am. Meat Inst. Found. Circ. No. 45.* 13.

Watts, B. M., J. Kendrick, M. W. Zipser, B. K. Hutchins, and B. Saleh. 1966. Enzymatic reducing pathways in meat. *J. Food Sci.* **31**, 855.

Younathan, M. T., and B. M. Watts. 1959. Relationship of meat pigments to lipid oxidation. *J. Food Sci.* **24**, 728.

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